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Pleiotrophin triggers inflammation and increased peritoneal permeability leading to peritoneal fibrosis

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Long-term peritoneal dialysis induces peritoneal fibrosis with submesothelial fibrotic tissue. Although angiogenesis and inflammatory mediators are involved in peritoneal fibrosis, precise molecular mechanisms are undefined. To study this, we used microarray analysis and compared gene expression profiles of the peritoneum in control and chlorhexidine gluconate (CG)-induced peritoneal fibrosis mice. One of the 43 highly upregulated genes was pleiotrophin, a midkine family member, the expression of which was also upregulated by the solution used to treat mice by peritoneal dialysis. This growth factor was found in fibroblasts and mesothelial cells within the underlying submesothelial compact zones of mice, and in human peritoneal biopsy samples and peritoneal dialysate effluent. Recombinant pleiotrophin stimulated mitogenesis and migration of mouse mesothelial cells in culture. We found that in wild-type mice, CG treatment increased peritoneal permeability (measured by equilibration), increased mRNA expression of TGF-β1, connective tissue growth factor and fibronectin, TNF-α and IL-1β expression, and resulted in infiltration of CD3-positive T cells, and caused a high number of Ki-67-positive proliferating cells. All of these parameters were decreased in peritoneal tissues of CG-treated pleiotrophin-knockout mice. Thus, an upregulation of pleiotrophin appears to play a role in fibrosis and inflammation during peritoneal injury.

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KEYWORDS: continuous ambulatory peritoneal dialysis; microarray analysis; peritoneal dialysis; peritoneal membrane

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Continuous ambulatory peritoneal dialysis (PD) is a preferred method of home dialysis for patients with end-stage renal failure.[1] Long-term use of PD induces peritoneal fibrosis characterized with the presence of submesothelial fibrotic tissue and increased peritoneal vascularization with vasculopathy.[2] Peritoneal fibrosis occurs in long-term continuous ambulatory PD patients in response to a variety of injuries, including bioincompatible dialysate solutions, peritonitis, uremia, and chronic inflammation.[2,3] Previous reports show that several profibrotic and proinflammatory mediators are upregulated upon induction of peritoneal fibrosis, such as transforming growth factor-β (TGF-β) and interleukin-6 (IL-6).[4–6] Although proinflammatory, angiogenic, and profibrotic cytokines such as IL-1β, vascular endothelial growth factor, and TGF-β are presumed to be involved in the pathogenesis, precise molecular mechanisms that lead to peritoneal sclerosis and encapsulating peritoneal sclerosis are still elusive.[7–9] To identify the novel genes possibly involved in the development of peritoneal fibrosis, we compared gene expression profiles of the peritoneum in chlorhexidine gluconate (CG)-induced peritoneal fibrosis and control mice using microarray.

Microarray analysis is a powerful tool to identify novel genes and pathways involved in the development of peritoneal fibrosis. Although a few papers report microarray analysis for endothelial cells in rat peritoneal dialysate infusion model,[10] analysis for whole mouse gene sets over 39,000 transcripts has not been investigated yet. In this study, we performed microarray analysis using a mouse model of peritoneal fibrosis and selected the genes that changed greatly in peritoneal fibrosis and those that were also present in mesothelial cells. This approach can allow us to specify the genes associated with peritoneal injury.

Here we show that one of the highly upregulated secreted proteins during the development of peritoneal fibrosis is pleiotrophin (PTN). PTN is an 18-kDa secreted protein, belonging to the midkine family and has functions similar to

midkine.[11] The receptors for PTN are the receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), anaplastic lymphoma tyrosine kinase (ALK), and syndecan-3.[12] PTN has been shown to promote cell growth, migration, oncogenesis, and angiogenesis.[11] However, the role of PTN in peritoneal fibrosis remains unknown. To elucidate the role of PTN in peritoneal injury, we examined the PTN expression in a mouse model of peritoneal fibrosis and in human peritoneal biopsy samples. We also investigated the functional significance of PTN by using PTN-deficient mice.[13]

RESULTS

To screen novel genes involved in development of peritoneal fibrosis, we compared the gene expression profiles between phosphate buffered saline (PBS)-injected and CG-injected mice three times a week for 3 weeks. As a control, PBS-injected wild-type mice showed no peritoneal fibrosis. CG-injected mice showed marked thickened submesothelial peritoneal membrane compared with PBS-injected mice (Figure 1a, CG-injected mice: 228 μm vs. PBS-injected mice: 34 μm). We performed microarray analysis using parietal peritoneum in mice at 21 days after PBS and CG treatment. We identified genes differentially expressed between PBS- and CG-injected mice, which were also expressed in murine cultured mesothelial cells. Table 1 shows one downregulated and 43 upregulated genes that were expressed by eightfold or greater in CG-treated mice than that in PBS-treated wild-type mice, and which were expressed in the cultured mesothelial

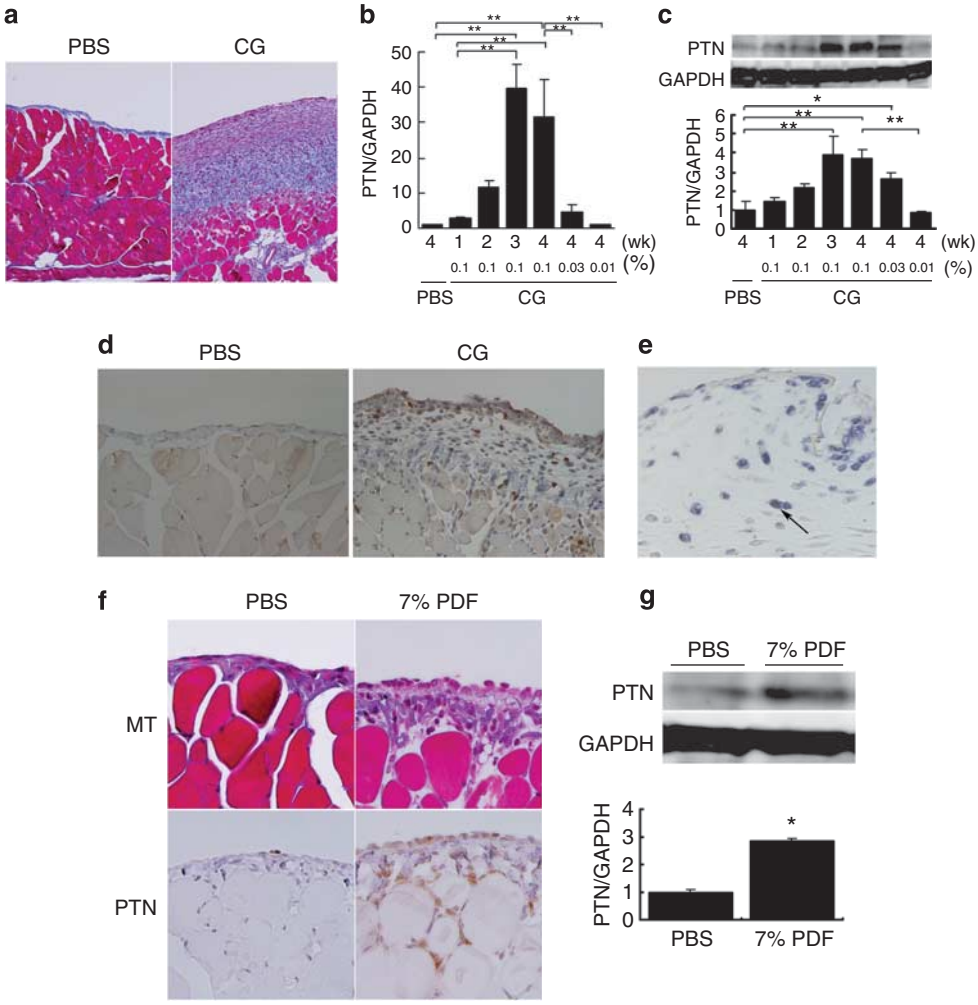

Figure 1 | PTN expression in a mouse model of peritoneal fibrosis. (**a**) Microscopic examination of peritoneal fibrosis model mice. C57BL/6J wild-type mice (WT) treated with phosphate-buffered saline (PBS) showed no fibrosis in the peritoneum. Chlorhexidine gluconate (CG)-treated mice exhibited marked peritoneal fibrosis with moderate infiltration of mononuclear cells on day 21 ($n = 3$, each, original magnification $\times 20$). Pleiotrophin (PTN) mRNA expression (**b**) or protein (**c**) in the peritoneum of PBS- or CG-treated mice was analyzed by real-time reverse transcriptase-polymerase chain reaction analysis or western blot analysis, respectively. GAPDH was used as internal control ($n = 5$, each). (**d**) Immunohistochemical study for PTN (brown). Mesothelial cells and the cells in submesothelial layer were positive for PTN. (**e**) Double immunohistochemical study for PTN (brown) and S100A4 (blue). Some of PTN-positive cells were also positive for S100A4 (arrow). (**f**) Mice receiving daily intraperitoneal injection of 7% peritoneal dialysis fluid (PDF) for 4 weeks showed increased submesothelial layer thickness by Masson's trichrome staining (MT) and upregulated PTN protein in the submesothelial layer ($n = 5$, each). (**g**) Western blot analysis showed that PTN protein in PDF-treated mice was 1.9 times higher than the control. GAPDH was used as internal control. Mean ± s.e. $^*P < 0.05$, $^{**}P < 0.01$ vs. PBS. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; wk, week.

Table 1 | Genes changed in parietal peritoneum in chlorhexidine gluconate (CG)-treated mice compared with phosphate-buffered saline-treated mice after 3 weeks of CG treatment and in the presence of cultured mesothelial cells

Gene title	ID	Fold change of gene up- or downregulated in CG-treated mice compared with PBS-treated mice	Gene symbol
Glucocortoid-regulated inflammatory prostaglandin GH synthase (griPGHS)	M94967	168.897	*Ptgs2*
Procollagen, type VIII, alpha 1	NM_007739	73.51669	*Col8a1*
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	55.71524	*Ddx3y*
Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	NM_012011	48.50293	*Eif2s3y*
A disintegrin and metallopeptidase domain 12 (meltrin alpha)	NM_007400	42.22425	*Adam12*
Interleukin 6	NM_031168	25.99208	*Il6*
Chemokine (C-X-C motif) ligand 1	NM_008176	24.25147	*Cxcl1*
Matrix metallopeptidase 14 (membrane-inserted)	NM_008608	24.25147	*Mmp14*
Chemokine (C-C motif) ligand 7	AF128193	17.14838	*Ccl7*
Ankyrin repeat domain 1 (cardiac muscle)	AK009959	16	*Ankrd1*
Leucine-rich repeat containing 15	AK017350	14.92853	*Lrrc15*
Chemokine (C-C motif) ligand 2	AF065933	14.92853	*Ccl2*
Interferon, alpha-inducible protein	AK019325	14.92853	*G1p2*
Runt-related transcription factor 1	NM_009821	12.12573	*Runx1*
Interferon regulatory factor 7	NM_016850	12.12573	*Irf7*
Collagen triple helix repeat containing 1	AK003674	12.12573	*Cthrc1*
Cytochrome P450, family 7, subfamily b, polypeptide 1	NM_007825	11.31371	*Cyp7b1*
Pleiotrophin	BC002064	11.31371	*Ptn*
Procollagen, type V, alpha 2	AV229424	11.31371	*Col5a2*
Fibronectin 1	BM234360	10.55606	*Fn1*
Chondroitin sulfate proteoglycan 2	NM_019389	10.55606	*Cspg2*
Thrombospondin 1	AI385532	10.55606	*Thbs1*
Membrane-spanning 4-domains, subfamily A, member 4C	NM_022429	10.55606	*Ms4a4c*
Lysyl oxidase	M65143	10.55606	*Lox*
Growth differentiation factor 15	NM_011819	9.849155	*Gdf15*
Dynamin 3, opposite strand	BB542096	9.849155	*Dnm3os*
RNA imprinted and accumulated in nucleus	BB649603	9.849155	*Rian*
WNT1-inducible signaling pathway protein 1	NM_018865	9.849155	*Wisp1*
Secreted frizzled-related sequence protein 1	BI658627	9.849155	*Sfrp1*
Procollagen, type III, alpha 1	AW550625	9.849155	*Col3a1*
Signal transducer and activator of transcription 2	AF088862	9.189587	*Stat2*
2′-5′ Oligoadenylate synthetase-like 2	BQ033138	9.189587	*Oasl2*
Tenascin C	NM_011607	9.189587	*Tnc*
Neural cell adhesion molecule 1	BB698413	8.574188	*Ncam1*
Integrin α5 (fibronectin receptor alpha)	BB493533	8.574188	*Itga5*
Tribbles homolog 3 (*Drosophila*)	BB508622	8.574188	*Trib3*
Gap junction membrane channel protein alpha 1	M63801	8.574188	*Gja1*
Interferon-induced protein with tetratricopeptide repeats 2	NM_008332	8.574188	*Ifit2*
Serine (or cysteine) peptidase inhibitor, clade A, member 3N	NM_009252	8.574188	*Serpina3n*
Lysyl oxidase-like 2	AF117951	8	*Loxl2*
GLI pathogenesis-related 2	BM208214	8	*Glipr2*
2′-5′ Oligoadenylate synthetase-like 1	AB067533	8	*Oasl1*
Tissue inhibitor of metalloproteinase 1	BC008107	8	*Timp1*
Immunoglobulin heavy chain 4 (serum IgG1)	BC008237	0.033493	*Igh4*

cells. Expression of extracellular matrix-related genes, including procollagen type VIII α1, was increased in peritoneal fibrosis model. Inflammatory cytokines including IL-6 were also upregulated. Among these upregulated genes, we focused on secreted proteins. One of them was PTN, which is an 18-kDa secreted protein and has been reported to promote mitogenesis and chemotaxis in cultured cells. Microarray analysis showed that PTN signal in the peritoneal membrane in the CG-injected wild-type mice was upregulated by 11-fold compared with PBS-injected mice (Table 1). Next, we confirmed the increase of PTN mRNA expression in the peritoneum of CG-treated mice by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Figure 1b). PTN mRNA in the peritoneum of CG-treated mice was gradually increased and peaked at 3 weeks by 39-fold compared with that in PBS-treated mice at 28 days, and was high until 4 weeks (Figure 1b). Diluted CG, such as 0.03 or 0.01%, induced weaker expression of PTN mRNA than 0.1% CG (Figure 1b). Western blot analysis also showed that PTN protein in the peritoneum of CG-treated mice was gradually upregulated and was highest at 3 weeks by 3.9-fold, as compared with that of PBS-treated mice (Figure 1c). A low concentration of 1:10 diluted CG induced less PTN expression (Figure 1c). Immunohistochemical study showed

that PTN was positive in the spindle-shaped cells and partly in mesothelial cells within the submesothelial layer (Figure 1d). Some of the spindle-shaped cells were also positive for a marker for fibroblasts S100A4, indicating that spindle-shaped cells were fibroblasts (Figure 1e). Next, we examined the effects of infusing peritoneal dialysis fluid (PDF) via a peritoneal catheter on expression of PTN. Mice receiving daily intraperitoneal injection of 7% PDF for 4 weeks showed increased PTN protein in the submesothelial layer with mild peritoneal fibrosis (Figure 1f). PTN protein in PDF-treated mice was 1.9 times higher than control mice, as observed by western blot analysis (Figure 1g).

We examined PTN expression in human biopsy samples at the insertion or removal of peritoneal catheters. PTN was expressed in human peritoneal biopsy samples by RT-PCR method (Figure 2a). Immunohistochemical study showed that PTN was located both in the mesothelial cells (arrows) and in the interstitial cells of peritoneal biopsy samples at the withdrawal from 5-year PD treatment (patient A), which was consistent with a mouse model of peritoneal fibrosis (Figure 2b). We examined whether peritoneal dialysate effluent contained PTN. Western blot analysis showed that peritoneal dialysates from six patients contained 15- and 18-kDa PTN (Figure 2c). The main form of PTN in peritoneal dialysate was 15 kDa. Patient B was a 54-year-old woman suffering from nephrosclerosis with a 2-year PD duration, patient C was a 47-year-old man suffering from diabetic nephropathy with a 2-year PD duration, and patient D was a 29-year-old woman suffering from immunoglobulin A nephropathy with a 1-year PD duration. Next, we examined PD effluent from patients with peritonitis. Patients E and F were 80- and 86-year-old women suffering from peritonitis by *Staphylococcus aureus*, respectively. PD effluents from patients with peritonitis tended to be high PTN levels. These results indicate that PTN exists in human peritoneal membrane and is detectable in peritoneal dialysate effluent.

Next, we investigated functional roles of PTN in cultured mouse peritoneal mesothelial cells. PTN exerts its effect, such as proliferation and chemotaxis, by binding its receptors, RPTPβ/ζ, ALK, and syndecan-3. We examined expression of Ptprz1, which encodes RPTPβ/ζ, ALK, and syndecan-3 in cultured mesothelial cells. Ptprz1 mRNA expression was detected in mesothelial cells but not in fibroblasts, macrophage cell line RAW264.7, mouse T-lymphoma cell line BW5147, mouse B-cell leukemia cell line BCL1-B20, or endothelial cell line bEnd.3 by real-time RT-PCR analyses (Figure 3a). Syndecan-3 mRNA expression was high in mesothelial cells and was also positive in fibroblasts, macrophages, and endothelial cells (Figure 3b). ALK mRNA expression was not detectable. PTN (1 ng/ml) stimulated proliferation in cultured mesothelial cells by 1.7-fold compared with vehicle-treated cells (Figure 3c). Angiotensin II and platelet-derived growth factor-BB stimulation showed less potent activity in cell proliferation than PTN, and endothelial growth factor treatment (100 ng/ml) revealed a similar potency to PTN stimulation (Figure 3c). PTN also induced mesothelial cell migration in the analysis of modified Boyden chamber method by threefold, as compared with vehicle-treated cells (Figure 3d). *In vitro*, fibroblasts cell line NIH3T3 fibroblasts showed higher PTN expression than mesothelial cells. RAW 264.7, mouse BW5147, BCL1-B20, and bEnd.3 showed virtually no expression of PTN (Figure 3e). These results indicate that PTN produced by fibroblasts may exert its biological effect on peritoneal mesothelial cells in the process of peritoneal fibrosis.

Finally, we examined whether PTN has a crucial role in peritoneal fibrosis progression. PTN knockout mice were treated with CG three times a week for 4 weeks. Although the thickness of peritoneal membrane in PTN knockout mice was similar compared with wild-type mice (Figure 4a and b), the expression of tumor necrosis factor-α and IL-1β mRNA was significantly reduced in CG-injected PTN knockout mice at 4 weeks, suggesting that PTN was involved in the inflammatory process (Figure 4c). Furthermore, gene expression of profibrotic factors, TGF-β1, connective tissue growth factor in CG-injected PTN knockout mice was reduced compared with CG-injected wild-type mice (Figure 4c). Fibronectin and type I collagen α1 chain were also decreased in CG-treated

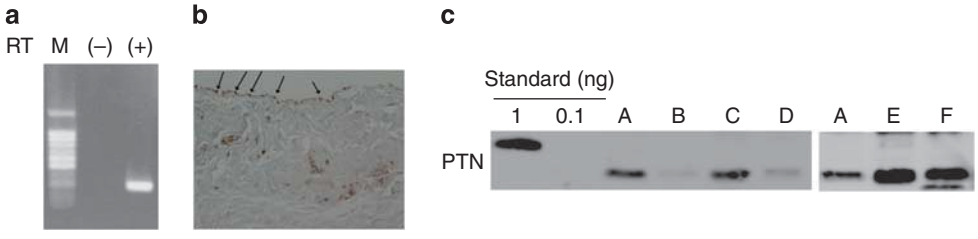

Figure 2 | PTN expression in human peritoneum and in peritoneal dialysate effluent. (**a**) Pleiotrophin (PTN) mRNA expression is detected by reverse transcriptase-polymerase chain reaction in human peritoneal biopsy sample. M, DNA marker (100 bp DNA ladder), RT (−) reverse transcriptase (−), RT (+) reverse transcriptase (+). (**b**) Immunohistochemical study for PTN in the peritoneal biopsy sample from a 5-year peritoneal dialysis (PD) patient A. (**c**) Western blot analysis for PTN in peritoneal dialysate effluent. Patient B was a 54-year-old woman suffering from nephrosclerosis with a 2-year PD duration, patient C was a 47-year-old man suffering from diabetic nephropathy with a 2-year PD duration, and patient D was a 29-year-old woman suffering from immunoglobulin A nephropathy with a 1-year PD duration. Patients E and F suffered from peritonitis by *Staphylococcus aureus*, and their PD efferents were shown on the first day of the peritonitis. Patient E was an 80-year-old woman suffering from nephrosclerosis with a 3-year PD duration. Patient F was an 86-year-old woman suffering from diabetic nephropathy with a 4-year PD duration.

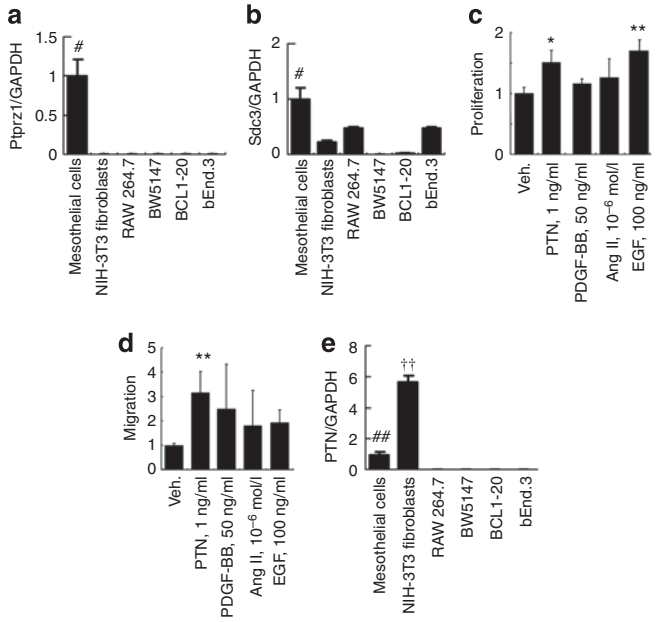

Figure 3 | PTN, Ptprz1, and syndecan-3 expression in cultured cells. The effect of pleiotrophin (PTN) on cell proliferation and migration in cultured mesothelial cells. (**a**) Ptprz1 mRNA expression was quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in cultured mesothelial cells, NIH3T3 fibroblasts, RAW264.7, BW5147, BCL1-20, and bEnd.3 cells ($n = 6$, each). (**b**) Syndecan-3 (Sdc3) mRNA expression was quantified by real-time RT-PCR in cultured mesothelial cells, NIH3T3 fibroblasts, RAW264.7, BW5147, BCL1-20, and bEnd.3 cells ($n = 6$, each). (**c**) The effect of PTN on cell proliferation in cultured mesothelial cells. Mesothelial cells were treated with PTN (1 ng/ml), platelet-derived growth factor (PDGF)-BB (50 ng/ml), angiotensin II (Ang II, 10^{-6} mol/l), endothelial growth factor (EGF, 100 ng/ml), or vehicle (Veh.). ^{3}H-thymidine incorporation was assessed ($n = 6$, each). (**d**) The effect of PTN on cell migration in cultured mesothelial cells by modified Boyden chamber method. Mesothelial cells were treated with PTN (1 ng/ml), PDGF-BB (50 ng/ml), angiotensin II (10^{-6} mol/l), or EGF (100 ng/ml) ($n = 6$, each). (**e**) PTN mRNA expression was quantified by real-time RT-PCR in cultured mesothelial cells, NIH3T3 fibroblasts, RAW264.7, BW5147, BCL1-20, and bEnd.3 cells ($n = 6$, each). Mean ± s.e. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. NIH3T3 fibroblasts, BW264.7, BW5147, BCL1-20 or bEnd.3. $^{\dagger\dagger}P < 0.01$ vs. mesothelial cells, RAW264.7, BW5147, BCL1-20 or bEnd.3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PTN knockout mice. On the other hand, expression of type IV collagen α1 chain was increased with the CG injection and was similar between wild-type and PTN knockout mice with CG treatment (Figure 4c). Macrophage infiltration was assessed by immunohistochemical study for F4/80. The number of macrophages in the peritoneum was increased in CG-injected wild-type mice. The number tended to decrease, but not significantly altered in CG-injected PTN knockout mice at 4 weeks (Figure 5a–d and q). In contrast, the number of CD3-positive T cells per the number of total cells in submesothelial area in CG-treated PTN knockout mice was significantly reduced compared with that in CG-treated wild-type mice at 4 weeks (5.6 ± 0.9 vs. 10.3 ± 0.5; Figure 5e–h and r). The effect of PTN on cell proliferation was evaluated by immunohistochemical study for Ki-67, a marker for cell proliferation (Figure 5i–l and s). Interestingly, immunohistochemical study showed that Ki-67-positive cells were localized within submesothelial compact zone and that cells positive for Ki-67 were presumed to be fibroblast-like cells according to their morphological appearance in CG-treated wild-type mice. The number of Ki-67-positive cells in CG-treated PTN-deficient mice was significantly decreased compared with that in CG-treated wild-type mice (1.5 ± 1.1 vs. 3.8 ± 1.2). Collagen IV deposition was similar between CG-treated wild-type mice and CG-treated PTN-deficient mice (Figure 5m–p). Peritoneal equilibration test was conducted to examine the functional role of PTN on peritoneal fibrosis at 2 weeks after the first CG injection. Figure 6 showed that the ratio of creatinine concentrations in the dialysate multiplied by dialysate volume over the plasma creatinine (D Cr × volume/P Cr) in PBS-treated PTN-deficient mice was not different from that in PBS-treated wild-type mice. In contrast, D Cr × volume/P Cr in CG-treated PTN-deficient mice was lower than that in CG-treated wild-type mice (1.93 ± 0.15 vs. 2.33 ± 0.38), suggesting that PTN deficiency was associated with low peritoneal transport in peritoneal fibrosis.

These results suggest that PTN has a crucial role in cell proliferation, extracellular matrix production, and peritoneal permeability during the development of peritoneal fibrosis through inflammatory process.

DISCUSSION

In this study, we identify for the first time that PTN is expressed in peritoneal tissues in mice and humans, especially in experimental peritoneal fibrosis mouse model. Mice receiving intraperitoneal infusion of PDF also showed increased expression of PTN, suggesting that PTN could be involved in peritoneal injury induced by dialysis solution. PTN is an 18- or 15-kDa heparin-binding protein.[11,14] PTN is initially identified as a neurite growth/guidance-regulating protein and belongs to the midkine family.[15,16] PTN has diverse functions including proliferation, mitogenic activities, apoptosis, oncogenic activity, and angiogenic activity.[11] PTN has been shown to have an important role in embryogenesis and kidney development.[17] PTN-deficient mice have been shown to have lower threshold for induction of long-term potentiation in hippocampal slices.[18] Other reports show that PTN-deficient mice exhibit less migration of neutrophils and macrophages to liver after partial hepatectomy,[19] and that female mice deficient in both midkine and PTN are infertile.[13]

The long-term PD can cause deterioration of the peritoneum,[20] and is closely associated with high peritoneal transport rate, which is one of the risk factors for developing encapsulating peritoneal sclerosis.[21] To evaluate the degree of peritoneal damage, several biomarkers have been investigated. Dialysate concentrations of IL-6 and vascular endothelial growth factor have been shown to be associated with increased peritoneal transport rate.[4] Dialysate fibrinogen/

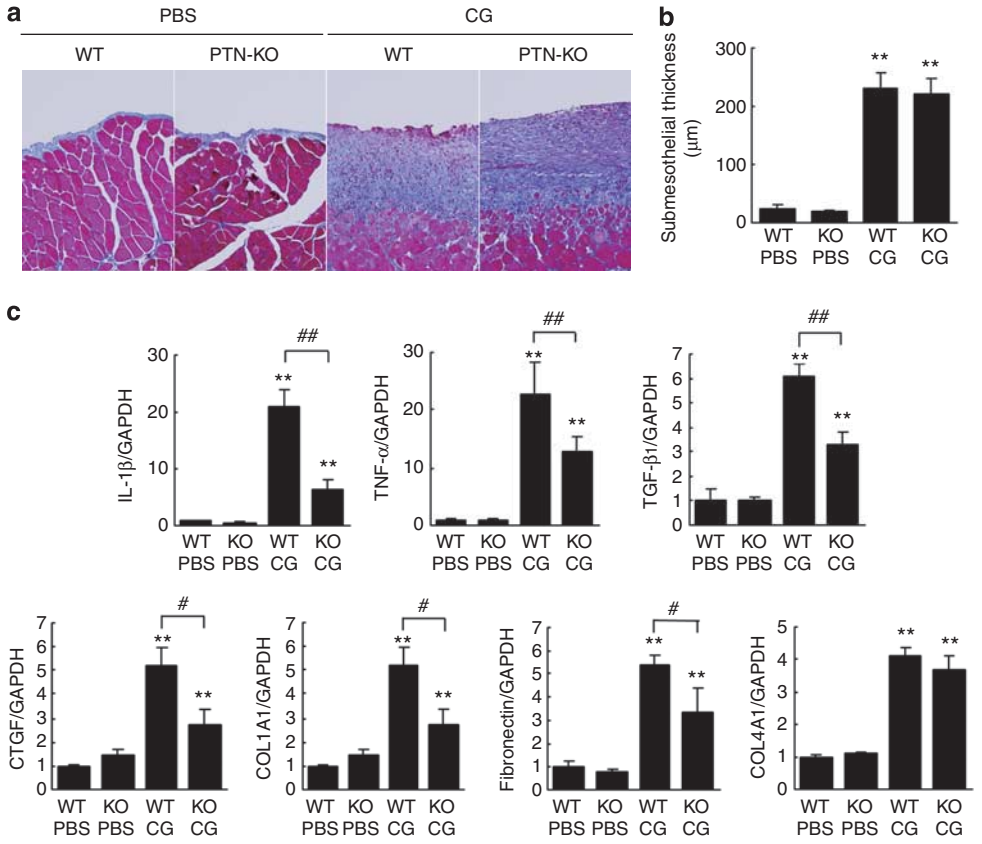

Figure 4 | Histological appearance and mRNA expression of peritoneum in PTN knockout mice (C57BL/6J background).[13] Pleiotrophin (PTN) knockout mice were treated with chlorhexidine gluconate (CG) three times a week for 4 weeks. (**a**) Masson's trichrome staining of peritoneum in wild-type or PTN knockout mice treated with phosphate-buffered saline (PBS) or CG. (**b**) The thickness of peritoneal membrane in mice. (**c**) Real-time reverse transcriptase-polymerase chain reaction analyses of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, connective tissue growth factor (CTGF), fibronectin, α1(I) collagen (COL1A1), and a1(IV) collagen (COL4A1). GAPDH was used as control. WT PBS: $n=5$, KO PBS: $n=4$, WT CG: $n=11$, KO CG: $n=10$. Mean ± s.e. $^{**}P<0.01$ vs. PBS-treated mice with the same genotype. $^{\#}P<0.05$, $^{\#\#}P<0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KO, PTN knockout mice; WT, wild-type mice.

fibrin degradation products also correlate with increased peritoneal permeability.[22] IL-6 is an acute-phase inflammatory reaction protein. The dialysate and ascite levels of inflammatory and fibrinolysis markers have been reported to increase before the development of encapsulating peritoneal sclerosis.[23] Although levels of inflammatory, angiogenic, and fibrinolytic markers such as IL-6, vascular endothelial growth factor, and fibrin degradation products could be important biomarkers for developing encapsulating peritoneal sclerosis, key molecules involved in the process of peritoneal damage are still elusive. In this study, we performed microarray analysis to identify genes differentially expressed between PBS-treated and CG-treated mice and expressed in cultured mesothelial cells. *Procollagen type VIII α1* gene and IL-6 were upregulated by 73.5- and 25.9-fold, respectively. These genes have been already reported under peritoneal damage, indicating that our microarray analysis is consistent with previous reports.[4,24] Genes identified by microarray analysis can be candidate ones for elucidating the development and progression of peritoneal fibrosis. In this study, we detected increased PTN mRNA expression and protein levels by 42- and 4-fold, respectively. The discrepancy between mRNA and protein levels may come from long half-life of the protein.[25] Our study revealed that human peritoneal biopsy samples contained PTN mRNA and protein. PTN protein in human peritoneum was located at mesothelial cells and fibroblasts, consistent with mouse peritoneal fibrosis model. We also revealed that PTN was detected in overnight-dwell peritoneal dialysate and that the main form of PTN in peritoneal dialysate is 15 kDa. Previously, Lu *et al.*[14] have demonstrated that PTN15 promotes glioblastoma proliferation in an ALK-dependent manner, whereas PTN18 promotes migration in a Ptprz1-dependent manner. Peritoneal dialysates from patients with peritonitis may contain high levels of PTN.

PTN mRNA expression was increased in peritoneal fibrosis model mice mainly at fibroblasts. *In vitro* study also showed that PTN expression was abundant in cultured fibroblasts compared with cultured mesothelial cells, lymphocytes, and macrophages. PTN gene expression has been shown to be upregulated in NIH3T3 fibroblasts stimulated by

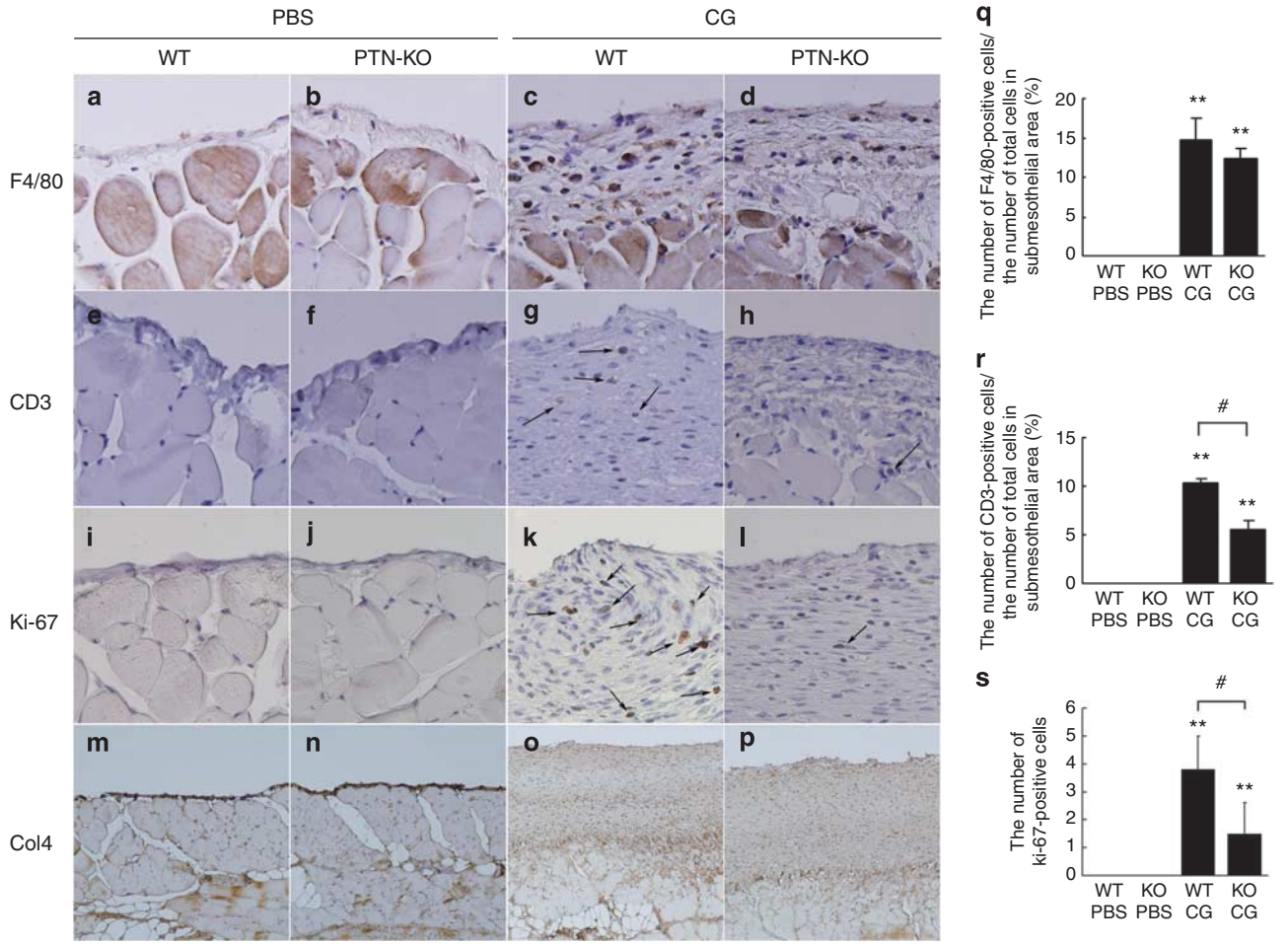

Figure 5 | Immunohistochemical study of F4/80, CD3, Ki-67, and type IV collagen (Col4). Mice were treated with chlorhexidine gluconate (CG) three times a week for 4 weeks. (**a**–**d**) F4/80, (**e**–**h**) CD3, (**i**–**l**) Ki67, and (**m**–**p**) Col4 staining in the peritoneum from (**a**, **e**, **i**, and **m**) phosphate-buffered saline (PBS)-injected wild-type (WT) mice, (**b**, **f**, **j**, and **n**) PBS-injected PTN knockout (KO) mice, (**c**, **g**, **k**, and **o**) CG-injected WT mice, (**d**, **h**, **l**, and **p**) CG-injected pleiotrophin (PTN)-KO mice. Arrows indicate positive cells. (**q**) The number of F4/80-positive cells per the number of total cells in the submesothelial area in mice. (**r**) The number of CD3-positive cells per the number of total cells in the submesothelial area in mice. (**s**) The number of Ki67-positive proliferated cells in the submesothelial area in mice. WT PBS: $n = 5$, KO PBS: $n = 4$, WT CG: $n = 11$, KO CG: $n = 10$. Mean ± s.e. $^{**}P < 0.01$ vs. PBS-treated mice with the same genotype in Figure 5. $^{\#}P < 0.05$.

platelet-derived growth factor-AB.[26] A recent paper reveals that PTN expression is strongly associated with IFN-γ/JAK/STAT1 signaling.[27] Further investigations are necessary to elucidate the molecular mechanism of PTN induction. PTN is a ligand of the RPTPβ/ζ,[12,28] ALK[12,] and syncecan-3.[12] Our study showed that RPTPβ/ζ was expressed in mesothelial cells, not in cultured fibroblasts, lymphocytes, or macrophages, and that syndecan-3 highly existed in mesothelial cells and was weakly expressed in fibroblasts and macrophages. ALK[29] was not detected in these cells. Taken together, PTN secreted by fibroblasts in submesothelial layer can have an effect on proliferation and migration mainly in mesothelial cells that express RPTPβ/ζ and syndecan-3.[30] The role of PTN receptors in peritoneal fibrosis needs further clarification.

In this study, the role of PTN in peritoneal fibrosis was investigated by using PTN-deficient mice. Without PTN, inflammatory and profibrotic responses were significantly reduced at 4 weeks after CG treatment, suggesting that PTN aggravates inflammation and fibrosis in the development of peritoneal fibrosis. Increased peritoneal permeability, examined by peritoneal equilibration tests, in CG-treated wild-type mice was almost completely ameliorated in CG-treated PTN knockout mice, suggesting that PTN can increase peritoneal permeability. Although CG-injected PTN knockout mice showed lower expression of COL1A1 and fibronectin than wild-type mice, the thickness of peritoneal membranes did not change between PTN-deficient mice and wild-type mice. The reason may come from similar expression levels of some other types of extracellular matrix such as collagen IV. Type IV collagen is abundantly deposited in peritoneal membrane.[31,32] Although macrophage infiltration has been shown to be an important phenomenon in peritoneal fibrosis,[33] CG-treated PTN-deficient mice showed no apparent difference in macrophage infiltration compared with wild-type mice. T cells are also an important part of

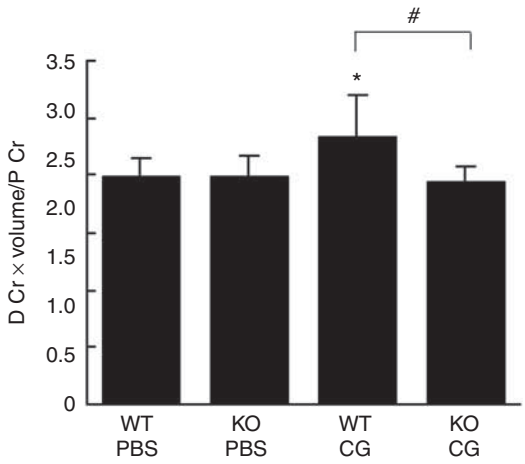

Figure 6 | Modified peritoneal equilibration test (PET). D Cr × volume/P Cr represents the creatinine (Cr) level of 7% glucose dialysate effluent (D) multiplied by volume divided by that of plasma (P) level in mice at 2 h retention. Chlorhexidine gluconate (CG)-injected wild-type (WT) mice showed increased D Cr × volume/P Cr compared with phosphate-buffered saline (PBS)-injected mice. Pleiotrophin (PTN)-knockout (KO) mice treated with CG showed reduced D Cr × volume/P Cr level compared with WT mice with CG. WT PBS: $n = 3$, KO PBS: $n = 6$, WT CG: $n = 5$, KO CG: $n = 7$. Mean ± s.e. $^{*}P < 0.05$ vs. PBS-treated mice with the same genotype in Figure 6. $^{\#}P < 0.05$.

peritoneal membrane damage,[34,35] and our results showed that T-cell infiltration was reduced in CG-treated PTN-deficient mice. PTN has been shown to induce expression of inflammatory cytokines in peripheral blood mononuclear cells.[36] The mechanism of T-cell infiltration by PTN is not clear, because RPTPβ/ζ mRNA nor syndecan-3 are not detected in cultured T-cell line. Downstream mediators of PTN need to be investigated in the future study. Cell proliferation was assessed by Ki-67 immunostaining.[37] PTN-deficient mice showed lower expression of Ki-67, indicating that cell proliferation in submesothelial layer was inhibited without PTN.

In conclusion, this study shows that PTN expression is upregulated in a mouse model of peritoneal fibrosis and is present in human peritoneal tissues and in peritoneal dialysate effluent, and that PTN secreted by fibroblasts or mesothelial cells can have a proliferative and chemotactic effect on mesothelial cells, and that PTN-deficient mice exhibit a weaker peritoneal membrane damage. These findings can be a help to elucidate a novel pathway in peritoneal fibrosis and suggest that PTN could be a promising biomarker against peritoneal damage.

MATERIALS AND METHODS

Patients

Patients who were admitted to Kyoto University Hospital for the diagnosis and treatment of renal disorders were enrolled under informed consent. This study was approved by the ethics committee on human research of Kyoto University Graduate School of Medicine. The parietal peritoneal samples were taken from the insertion site of a PD catheter located in the lumbar region as biopsies at the beginning or ending of PD. Peritoneal dialysate effluents were obtained at the time of the exchange. Expression of PTN in peritoneal biopsy sample was assessed by the RT-PCR method. After RNA extraction by RNeasy mini kit (Qiagen, Valencia, CA), complementary DNA was generated using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. RT-PCR was performed using the following primers: forward, 5′-gggaagaaagagaccagtgagt-3′ and reverse, 5′-ctggtttctctttcttccctgc-3′.

Induction of peritoneal fibrosis and phenotypic analysis

All animal experiments were approved by the animal experimentation committee of Kyoto University Graduate School of Medicine. Peritoneal fibrosis was induced in mice with the intraperitoneal injections of 0.3 ml of 0.1% CG in 15% ethanol and 85% PBS three times a week for 1, 2, 3, and 4 weeks as previously reported ($n = 5$, each).[38] For evaluating dose–response, mice were administered 0.3 ml of 0.01% and 0.03% CG in 15% ethanol and 85% PBS three times a week for 4 weeks. Control mice received intraperitoneal injection of PBS. Mice were killed under pentobarbital anesthesia, and peritoneal tissues were obtained from the upper portion of the parietal peritoneum to avoid injured peritoneum by repeated injections.

For dialysis fluid infusion, silicone port catheters with two cuffs were used (PennyPort MMP-4S; Access Technologies, Skokie, IL). After mice were anesthetized under pentobarbital, an incision was made in the skin of the back and left lumbar portion. Peritoneal membrane was pricked with a 20-Gy needle to make a small hole. The catheter port was implanted under the skin of the back and the catheters were inserted along the needle hole. A catheter port was placed subcutaneously on the back. One milliliter of PDFs (Perisate; JMS, Hiroshima, Japan) containing 7% PD solution or PBS were administered intraperitoneally via a catheter port using a 26-Gy needle every day for 4 weeks ($n = 5$, each). PTN-deficient mice (C57BL/6J background) were generated as described previously.[13]

Modified peritoneal equilibration test

Modified peritoneal equilibration test was conducted to determine the peritoneal permeability. Wild-type or PTN knockout mice were injected with PBS or CG ($n = 5$, each) three times for 2 weeks, and were then administered intraperitoneal injection of 3 ml of 7% glucose dialysis solution (Perisate; JMS). After 2 h of retention, dialysis fluids were collected and blood samples were withdrawn. Serum and dialysate creatinine levels were measured by using the enzymatic method (SRL, Tokyo, Japan).

Affymetrix gene chip array

Wild-type mice were subjected to peritoneal fibrosis with the intraperitoneal injections of 0.3 ml of 0.1% CG in 15% ethanol and 85% PBS three times a week for 3 weeks ($n = 3$). As control, PBS-treated wild-type mice were used ($n = 3$). Total RNA from the parietal peritoneum at day 21 was extracted by RNeasy Mini Kit (Qiagen) in these mice.[39] Complementary RNA probes were generated using the GeneChip Expression 3′-Amplification Reagents for IVT Labeling Kit (Applied Biosystems, Foster city, CA) and each sample was hybridized to an Affymetrix mouse genome 430 2.0 array at TAKARA Bio (Shiga, Japan). After washing, the genechips were scanned by a GeneChip Scanner 3000. Data normalization, log transformation, statistical analysis, and pattern study were performed with the GeneChip Operating Software.

Histology and immunohistochemistry

Peritoneal membrane sections were fixed with 4% buffered paraformaldehyde and embedded in paraffin. Sections (1 μm thick) were stained with Masson's trichrome.[40] We measured the thickness of the fibrotic submesothelial zone above the abdominal muscle layer in cross-sections as described previously,[41] by using MetaMorph software (Molecular Devices, Downingtown, PA). Ten different points were examined by two investigators without knowledge of the origin of the slides. The results were expressed as the average peritoneal membrane thickness. For immunohistochemical analyses of PTN, collagen type IV, F4/80, CD3, and Ki-67 in mice, the sections were processed as described.[40] After antigen retrieval, the samples were incubated with rabbit polyclonal anti-PTN antibody (ProteinTech Group, Chicago, IL), rabbit polyclonal anti-mouse collagen type IV antibody (Millipore, Billerica, MA), rat monoclonal anti-F4/80 antibody (Serotec, Oxford, UK), rabbit polyclonal anti-CD3 antibody (DAKO, Glostrup, Denmark), and rat monoclonal anti-Ki-67 antibody (DAKO). After incubation with horseradish peroxidase-conjugated secondary antibodies, the specimens were developed using 3,3′-diaminobenzidine tetrahydrochloride. For immunohistochemical study of human PTN, we used a rabbit polyclonal anti-PTN antibody (Abcam, Cambridge, UK) as a primary antibody.

Real-time PCR analysis

Quantitative real-time PCR was performed using Premix Ex Taq (TAKARA Bio) on an Applied Biosystems 7300 real-time PCR system (Applied Biosystems) or a StepOnePlus system (Applied Biosystems), as described previously with some modification.[39] To determine mouse PTN, Ptprz1, TGF-β1, connective tissue growth factor, COL1A1, COL4A1, fibronectin, and IL-1β and tumor necrosis factor-α expression levels, gene-specific primers and probes were used. Primers and probe sequences are listed in Supplementary Table S1 online. Expression of each mRNA was normalized for glyceraldehyde-3-phosphate dehydrogenase using TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase control reagents (Applied Biosystems).

Western blot analysis

Western blot analysis was performed as described.[39] Filters transferred onto protein extracts or peritoneal dialysate effluents were incubated with rabbit polyclonal anti-PTN antibody (ProteinTech Group) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were then developed using a chemiluminescence kit (GE healthcare, Piscataway, NJ).

Cell culture

Mouse peritoneal mesothelial cells were obtained using a standard trypsin/ethylenediaminetetraacetic acid digestion method from the peritoneal wall of adult male C57BL/6J mice.[42,43] The excised peritoneal flap was cut into small pieces and then incubated, with constant agitation, with 0.25% trypsin and 1 mmol/l ethylenediaminetetraacetic acid (Invitrogen) for 15 min at 37 °C. The released cells were centrifuged at 1200 r.p.m. for 5 min and cultured with Dulbecco's Modified Eagle Medium (DMEM D6046; Sigma-Aldrich, St Louis, MI) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (25 ng/ml). NIH3T3 fibroblasts, RAW264.7 cells, and bEnd.3 cells were obtained from American Type Culture Collection (Manassas, VA). BW5147 cells were provided by Health Science Research Bank (Sennan, Osaka, Japan) and BCL1-B20 cells were provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. BCL1-B20 cells were cultured with RPMI1640 (Sigma) with 10% FBS. Other cells were cultured with DMEM with 10% FBS.

Proliferation assay in cultured mesothelial cells was performed with ^{3}H-thymidine as described previously.[44] Briefly, mesothelial cells were plated on 24-well plates ($n = 6$, each group) and incubated with DMEM containing 0.3% FBS for the 24 h. Cell proliferation was studied in the presence of 1 ng/ml of recombinant human PTN (R&D Systems, Minneapolis, MN), 10 ng/ml of recombinant human platelet-derived growth factor-BB (BD Biosciences, San Jose, CA), 10^{-6} mol/l of angiotensin II (Peptide Institute, Osaka, Japan), 100 ng/ml of recombinant human endothelial growth factor (PeproTech EC, London, UK), or vehicle (PBS) for 24 h with DMEM containing 0.3% FBS. ^{3}H-thymidine was added simultaneously with the above-described agents. Migration assay was performed as described previously.[45] In brief, migration of mesothelial cells was analyzed by modified Boyden chamber method using 96-well chemotaxis chambers. In the upper chambers, mesothelial cells were placed with DMEM containing 0.02% bovine serum albumin. In the lower chambers, there were serum-free DMEM containing PTN (1 ng/ml), platelet-derived growth factor-BB (50 ng/ml), angiotensin II (10^{-6} mol/l), or endothelial growth factor (100 ng/ml). The cells were incubated for 4 h and the filters were stained with 0.5% Coomassie Brilliant Blue R250 (Nacalai Tesque, Kyoto, Japan) in 50% methanol, 40% water, and 10% acetic acid ($n = 6$, each).

Statistical analysis

Data are expressed as the mean ± s.e. Statistical analysis was performed using one-way analysis of variance as appropriate. A P-value < 0.05 was considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. TaqMan primers and probe sequences.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Ledebo I, Ronco C. The best dialysis therapy? Results from an international survey among nephrology professionals. *NDT Plus* 2008; **1**: 403–408.
2. Saxena R. Pathogenesis and treatment of peritoneal membrane failure. *Pediatr Nephrol* 2008; **23**: 695–703.
3. Yung S, Chan TM. Preventing peritoneal fibrosis—insights from the laboratory. *Perit Dial Int* 2003; **23**(S2): S37–S41.
4. Pecoits-Filho R, Araujo MR, Lindholm B *et al.* Plasma and dialysate IL-6 and VEGF concentrations are associated with high peritoneal solute transport rate. *Nephrol Dial Transplant* 2002; **17**: 1480–1486.
5. Lai KN, Lai KB, Szeto CC *et al.* Growth factors in continuous ambulatory peritoneal dialysis effluent. Their relation with peritoneal transport of small solutes. *Am J Nephrol* 1999; **19**: 416–422.

6. Oh KH, Jung JY, Yoon MO *et al.* Intra-peritoneal interleukin-6 system is a potent determinant of the baseline peritoneal solute transport in incident peritoneal dialysis patients. *Nephrol Dial Transplant* 2010; **25**: 1639–1646.
7. Margetts PJ, Kolb M, Galt T *et al.* Gene transfer of transforming growth factor-β1 to the rat peritoneum: effects on membrane function. *J Am Soc Nephrol* 2001; **12**: 2029–2039.
8. Margetts PJ, Kolb M, Yu L *et al.* Inflammatory cytokines, angiogenesis, and fibrosis in the rat peritoneum. *Am J Pathol* 2002; **160**: 2285–2294.
9. Aroeira LS, Aguilera A, Selgas R *et al.* Mesenchymal conversion of mesothelial cells as a mechanism responsible for high solute transport rate in peritoneal dialysis: role of vascular endothelial growth factor. *Am J Kidney Dis* 2005; **46**: 938–948.
10. Zakaria el R, Matheson PJ, Hurt RT *et al.* Chronic infusion of sterile peritoneal dialysis solution abrogates enhanced peritoneal gene expression responses to chronic peritoneal catheter presence. *Adv Perit Dial* 2008; **24**: 7–15.
11. Deuel TF, Zhang N, Yeh HJ *et al.* Pleiotrophin: a cytokine with diverse functions and a novel signaling pathway. *Arch Biochem Biophys* 2002; **397**: 162–171.
12. Jin L, Jianghai C, Juan L *et al.* Pleiotrophin and peripheral nerve injury. *Neurosurg Rev* 2009; **32**: 387–393.
13. Muramatsu H, Zou P, Kurosawa N *et al.* Female infertility in mice deficient in midkine and pleiotrophin, which form a distinct family of growth factors. *Genes Cells* 2006; **11**: 1405–1417.
14. Lu KV, Jong KA, Kim GY *et al.* Differential induction of glioblastoma migration and growth by two forms of pleiotrophin. *J Biol Chem* 2005; **280**: 26953–26964.
15. Milner PG, Li YS, Hoffman RM *et al.* A novel 17 kD heparin-binding growth factor (HBGF-8) in bovine uterus: purification and N-terminal amino acid sequence. *Biochem Biophys Res Commun* 1989; **165**: 1096–1103.
16. Rauvala H. An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. *EMBO J* 1989; **8**: 2933–2941.
17. Sakurai H, Bush KT, Nigam SK. Identification of pleiotrophin as a mesenchymal factor involved in ureteric bud branching morphogenesis. *Development* 2001; **128**: 3283–3293.
18. Amet LE, Lauri SE, Hienola A *et al.* Enhanced hippocampal long-term potentiation in mice lacking heparin-binding growth-associated molecule. *Mol Cell Neurosci* 2001; **17**: 1014–1024.
19. Ochiai K, Muramatsu H, Yamamoto S *et al.* The role of midkine and pleiotrophin in liver regeneration. *Liver Int* 2004; **24**: 484–491.
20. Yamamoto R, Nakayama M, Hasegawa T *et al.* High-transport membrane is a risk factor for encapsulating peritoneal sclerosis developing after long-term continuous ambulatory peritoneal dialysis treatment. *Adv Perit Dial* 2002; **18**: 131–134.
21. Kawanishi H, Moriishi M. Encapsulating peritoneal sclerosis: prevention and treatment. *Perit Dial Int* 2007; **27**(S2): S289–S292.
22. Kawanishi H, Fujimori A, Tsuchida K *et al.* Markers in peritoneal effluent for withdrawal from peritoneal dialysis: multicenter prospective study in Japan. *Adv Perit Dial* 2005; **21**: 134–138.
23. Kawanishi H, Harada Y, Noriyuki T *et al.* Treatment options for encapsulating peritoneal sclerosis based on progressive stage. *Adv Perit Dial* 2001; **17**: 200–204.
24. Xu X, Rivkind A, Pappo O *et al.* Role of mast cells and myofibroblasts in human peritoneal adhesion formation. *Ann Surg* 2002; **236**: 593–601.
25. Dreyfus J, Brunet-de Carvalho N, Duprez D *et al.* HB-GAM/pleiotrophin: localization of mRNA and protein in the chicken developing leg. *Int J Dev Biol* 1998; **42**: 189–198.
26. Li YS, Gurrieri M, Deuel TF. Pleiotrophin gene expression is highly restricted and is regulated by platelet-derived growth factor. *Biochem Biophys Res Commun* 1992; **184**: 427–432.
27. Li F, Tian F, Wang L *et al.* Pleiotrophin (PTN) is expressed in vascularized human atherosclerotic plaques: IFN-γ/JAK/STAT1 signaling is critical for the expression of PTN in macrophages. *FASEB J* 2010; **24**: 810–822.
28. Meng K, Rodriguez-Pena A, Dimitrov T *et al.* Pleiotrophin signals increased tyrosine phosphorylation of β-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase β/ζ. *Proc Natl Acad Sci U S A* 2000; **97**: 2603–2608.
29. Stoica GE, Kuo A, Aigner A *et al.* Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. *J Biol Chem* 2001; **276**: 16772–16779.
30. Raulo E, Chernousov MA, Carey DJ *et al.* Isolation of a neuronal cell surface receptor of heparin binding growth-associated molecule (HB-GAM). Identification as N-syndecan (syndecan-3). *J Biol Chem* 1994; **269**: 12999–13004.
31. Mateijsen MA, van der Wal AC, Hendriks PM *et al.* Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. *Perit Dial Int* 1999; **19**: 517–525.
32. Saito H, Kitamoto M, Kato K *et al.* Tissue factor and factor V involvement in rat peritoneal fibrosis. *Perit Dial Int* 2009; **29**: 340–351.
33. Schilte MN, Celie JW, Wee PM *et al.* Factors contributing to peritoneal tissue remodeling in peritoneal dialysis. *Perit Dial Int* 2009; **29**: 605–617.
34. Glik A, Douvdevani A. T lymphocytes: the 'cellular' arm of acquired immunity in the peritoneum. *Perit Dial Int* 2006; **26**: 438–448.
35. Devuyst O, Margetts PJ, Topley N. The pathophysiology of the peritoneal membrane. *J Am Soc Nephrol* 2010; **21**: 1077–1085.
36. Achour A, M'Bika JB, Baudouin F *et al.* Pleiotrophin induces expression of inflammatory cytokines in peripheral blood mononuclear cells. *Biochimie* 2008; **90**: 1791–1795.
37. Endl E, Gerdes J. The Ki-67 Protein: Fascinating forms and an unknown function. *Exp Cell Res* 2000; **257**: 231–237.
38. Ishii Y, Sawada T, Shimizu A *et al.* An experimental sclerosing encapsulating peritonitis model in mice. *Nephrol Dial Transplant* 2001; **16**: 1262–1266.
39. Yokoi H, Mukoyama M, Mori K *et al.* Overexpression of connective tissue growth factor in podocytes worsens diabetic nephropathy in mice. *Kidney Int* 2008; **73**: 446–455.
40. Yokoi H, Mukoyama M, Nagae T *et al.* Reduction in connective tissue growth factor by antisense treatment ameliorates renal tubulointerstitial fibrosis. *J Am Soc Nephrol* 2004; **15**: 1430–1440.
41. Yoshio Y, Miyazaki M, Abe K *et al.* TNP-470, an angiogenesis inhibitor, suppresses the progression of peritoneal fibrosis in mouse experimental model. *Kidney Int* 2004; **66**: 1677–1685.
42. Tamura M, Osajima A, Nakayamada S *et al.* High glucose levels inhibit focal adhesion kinase-mediated wound healing of rat peritoneal mesothelial cells. *Kidney Int* 2003; **63**: 722–731.
43. Yung S, Li FK, Chan TM. Peritoneal mesothelial cell culture and biology. *Perit Dial Int* 2006; **26**: 162–173.
44. Suganami T, Mukoyama M, Sugawara A *et al.* Overexpression of brain natriuretic peptide in mice ameliorates immune-mediated renal injury. *J Am Soc Nephrol* 2001; **12**: 2652–2663.
45. Sawai K, Mori K, Mukoyama M *et al.* Angiogenic protein Cyr61 is expressed by podocytes in anti-Thy-1 glomerulonephritis. *J Am Soc Nephrol* 2003; **14**: 1154–1163.